

Integrin $\alpha 8 \beta 1$ Is Critically Important for Epithelial–Mesenchymal Interactions during Kidney Morphogenesis

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Summary

We present genetic evidence that integrins regulate epithelial–mesenchymal interactions during organogenesis. Mice with a mutation in the $\alpha 8$ gene do not express the integrin $\alpha 8 \beta 1$ and exhibit profound deficits in kidney morphogenesis. In wild-type animals, inductive interactions between the ureteric epithelium and metanephric mesenchyme are essential for kidney morphogenesis. In $\alpha 8$ mutant homozygotes, growth and branching of the ureteric bud and recruitment of mesenchymal cells into epithelial structures are defective. Consistent with these phenotypes, $\alpha 8$ expression is induced in mesenchymal cells upon contact with the ureter. Since none of its previously identified ligands appears likely to mediate the essential functions of $\alpha 8 \beta 1$ in kidney morphogenesis, we have used an $\alpha 8 \beta 1$ –alkaline phosphatase chimera to localize novel ligand(s) in the growing ureter. The distribution of these ligand(s) makes them strong candidates for regulators of kidney morphogenesis.

Introduction

The development of an organism requires precise spatiotemporal coordination of cell behavior and is therefore highly dependent on communication among cells. Reciprocal inductive interactions among cells or groups of cells are of particular importance during embryogenesis because they result in cellular differentiation and formation of tissues and organs (Spemann and Mangold, 1924; reviewed in Gurdon, 1992). Morphogenesis of the kidney serves as an excellent model system to analyze the cellular and molecular mechanisms underlying organogenesis (reviewed in Grobstein, 1956; Saxen, 1987). Kidney development depends on a series of reciprocal inductive events between the ureteric epithelium and the metanephric mesenchyme. In the mouse, the ureteric bud emanates from the Wolffian duct on embryonic day (E) 10.5–E11 (Figure 1). Subsequently, the ureteric bud grows toward and invades the metanephric mesenchyme. Mesenchymal cells condense around the tips of the growing and branching ureter and are transformed

in a sequential order into pretubular aggregates, renal vesicles, comma-shaped bodies, and S-shaped bodies that ultimately give rise to the epithelia of the tubules and glomeruli of the mature kidney. The many branches of the ureter form the collecting duct tree of the kidney.

Molecular genetic analyses in mice have revealed a complex network of regulatory proteins that control kidney organogenesis. These include the secreted signaling molecules glial cell line–derived neurotrophic factor (GDNF), wnt-4, bone morphogenetic protein 7 (BMP7), and platelet-derived growth factor B (PDGF-B) and their receptors such as the c-ret and PDGFR tyrosine kinases (Soriano, 1994; Leveen et al., 1994; Stark et al., 1994; Dudley et al., 1995; Luo et al., 1995; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1996). A number of transcription factors, including WT-1, Pax-2, and BF-2, are also required for kidney development (Kreidberg et al., 1993; Torres et al., 1995; Hatini et al., 1996). Transcription factors and signaling molecules must regulate expression levels and activities of downstream targets that execute the imposed developmental program. Among strong candidates for downstream targets are integrins, a family of heterodimeric cell surface proteins that serve as receptors for extracellular matrix (ECM) molecules and counterreceptors on adjacent cells (reviewed in Hynes, 1992). Experiments with kidney organ cultures have provided evidence that integrin receptors and their ligands regulate the development of polarized epithelia from metanephric mesenchymal cells following induction by the ureteric epithelium (reviewed in Ekblom, 1996).

We now demonstrate that integrins are required *in vivo* for kidney morphogenesis. We have previously shown that the integrin $\alpha 8$ subunit forms heterodimers exclusively with the integrin $\beta 1$ subunit and that these heterodimers serve as receptors for the ECM molecules fibronectin (FN), vitronectin (VN), and tenascin-C (TN-C) (Müller et al., 1995; Schnapp et al., 1995; Varnum-Finney et al., 1995). We show here that integrin $\alpha 8 \beta 1$ is expressed in many developing organs and particularly in the kidney, in mesenchymal cells bordering on epithelial cell sheets that undergo branching morphogenesis. Mice carrying a targeted mutation in the integrin $\alpha 8$ gene show severe deficits in kidney morphogenesis due to reduced growth of the ureteric bud toward the metanephric mesenchyme, reduced branching of the ureteric epithelium within the mesenchyme, and defective epithelialization of kidney mesenchymal cells. We colocalize a novel ligand to the surface of the branching ureter that is likely a mediator of the effects on kidney morphogenesis.

Results

Integrin $\alpha 8$ Expression Is Induced in Mesenchymal Cells Surrounding the Growing and Branching Ureter

To determine the expression patterns of the integrin $\alpha 8$ subunit during mouse development, we cloned the

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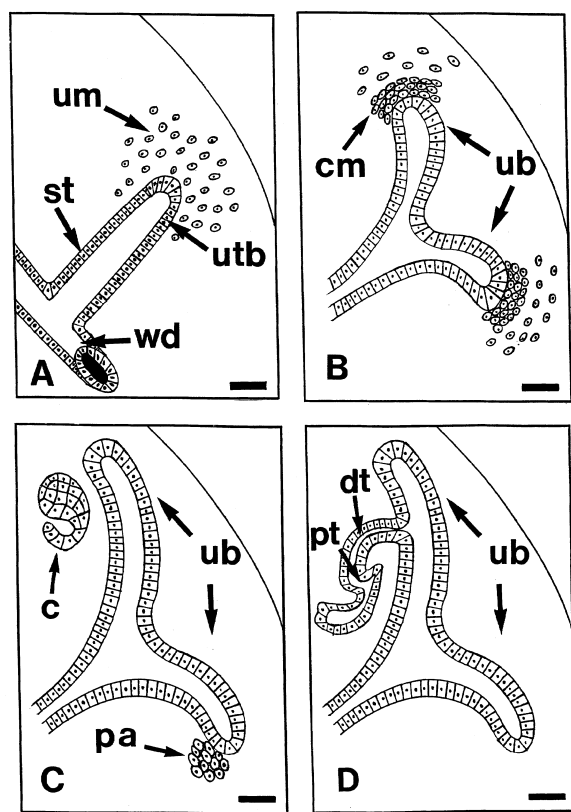


Figure 1. Schematic Representation of Kidney Development

(A) At E10.5–E11.5 the ureteric bud (utb) emanates from the Wolffian duct (wd).

(B) The ureteric bud invades the metanephric mesenchyme (um) and branches. The mesenchyme condenses (cm) around the tip of the ureter branches (ub).

(C) The mesenchyme is transformed via intermediates such as pretubular aggregates (pa) and comma-shaped bodies (c).

(D) The mesenchyme has transformed into an epithelium consisting of distal tubules (dt) and proximal tubules (pt) that will mature into the excretory nephron. st, ureter stalk.

Scale bars, 10 μ m.

mouse $\alpha 8$ cDNA and raised in rabbits an antibody against a peptide encompassing the cytoplasmic domain of $\alpha 8$. A second antibody was raised against the extracellular domain of the $\alpha 8$ subunit expressed as a soluble secreted protein in COS cells. Both antibodies were affinity purified, revealed identical expression patterns for the $\alpha 8$ subunit, and did not cross-react with other integrins in Western blots performed with extracts from various mouse tissues (data not shown). In agreement with earlier observations in chicken (Bossy et al., 1991), immunoprecipitation analyses employing extracts from various mouse tissues revealed that the $\alpha 8$ subunit was associated exclusively with the integrin $\beta 1$ subunit (data not shown). At all developmental stages analyzed, the integrin $\alpha 8$ subunit, reflecting the presence of the $\alpha 8\beta 1$ receptor, was highly expressed in mesenchymal but not epithelial cells in developing organs such as the gut, the lung, the gonads, and the nephrogenic cord (Figures 2 and 3 and data not shown).

The spatiotemporal expression patterns of the integrin $\alpha 8$ subunit in the developing kidney were studied in

detail. Figure 1 summarizes different stages of development of the metanephric kidney, and Figure 2 shows the corresponding expression patterns for the integrin $\alpha 8$ subunit. At E11, the integrin $\alpha 8$ subunit was expressed throughout the mesenchyme of the nephrogenic cord. Very high expression was observed in mesenchymal cells immediately surrounding the Wolffian duct (Figure 2A). Prior to formation of the ureteric bud, no $\alpha 8$ expression was evident within the mesenchyme that separates the urogenital ridge from the metanephric mesenchyme and within the metanephric mesenchyme itself. Upon branching of the ureteric bud from the Wolffian duct, expression of the integrin $\alpha 8$ subunit was induced in cells surrounding the growing ureteric epithelium as it extended toward the metanephric mesenchyme (Figures 2A and 2B). At E13.5, the $\alpha 8$ subunit was expressed within the metanephric mesenchyme in mesenchymal condensates around the branching tips of the ureter (Figures 2C and 2E) and in pretubular aggregates (Figure 2F) but not on comma-shaped bodies (Figure 2G) or S-shaped bodies (Figure 2F). Thus, expression of the $\alpha 8$ subunit was first induced in mesenchymal cells upon contact with the ureter but was subsequently down-regulated upon epithelialization of these cells. This was particularly evident in sections from E16.5 animals (Figure 2H), where $\alpha 8$ was expressed in condensing mesenchymal cells in the outer marginal zone of the kidney but not in more internal parts that contained more differentiated structures.

In contrast to the transient expression of the $\alpha 8$ subunit in mesenchymal cells that differentiated into epithelium, $\alpha 8$ expression remained high in the mesenchymal cells that bordered on the ureteric epithelium (Figures 2D and 2E). The strong polarized and continuous expression of the $\alpha 8$ subunit on the cell surface of mesenchymal cells facing toward the ureter epithelium suggests that $\alpha 8\beta 1$ heterodimers may become localized by a ligand expressed on the ureteric epithelium.

Integrin $\alpha 8$ -Deficient Mice Show at Birth Renal Agenesis or Dysgenesis

To determine the essential functions of the integrin $\alpha 8\beta 1$ receptor, we inactivated the gene encoding the $\alpha 8$ subunit by gene targeting in mice (Figure 3). Heterozygous animals carrying one mutated $\alpha 8$ locus showed no overt abnormal phenotype. Homozygous mutant animals developed to birth with approximately the expected Mendelian frequency (33 of 163 animals), but most homozygous mutants died by the first or second day after birth. Western blot analysis as well as immunohistochemistry confirmed that we had generated a null allele, because $\alpha 8$ protein was undetectable in homozygous mutants by either technique (Figures 3C and 3D).

Analysis of homozygous mutant animals at birth revealed severe kidney abnormalities (Figure 4 and Table 1). In contrast to wild-type animals (Figure 4A), 54% of the mutant animals were born without ureters or kidneys (Figure 4B). In 40% of the animals a ureter had formed unilaterally and had invaded the metanephric mesenchyme (Figures 4C and 4D). Once the ureter had invaded the mesenchyme, either it did not branch and no nephrons were evident (14% of the mutants), or more substantial branching occurred and excretory nephrons

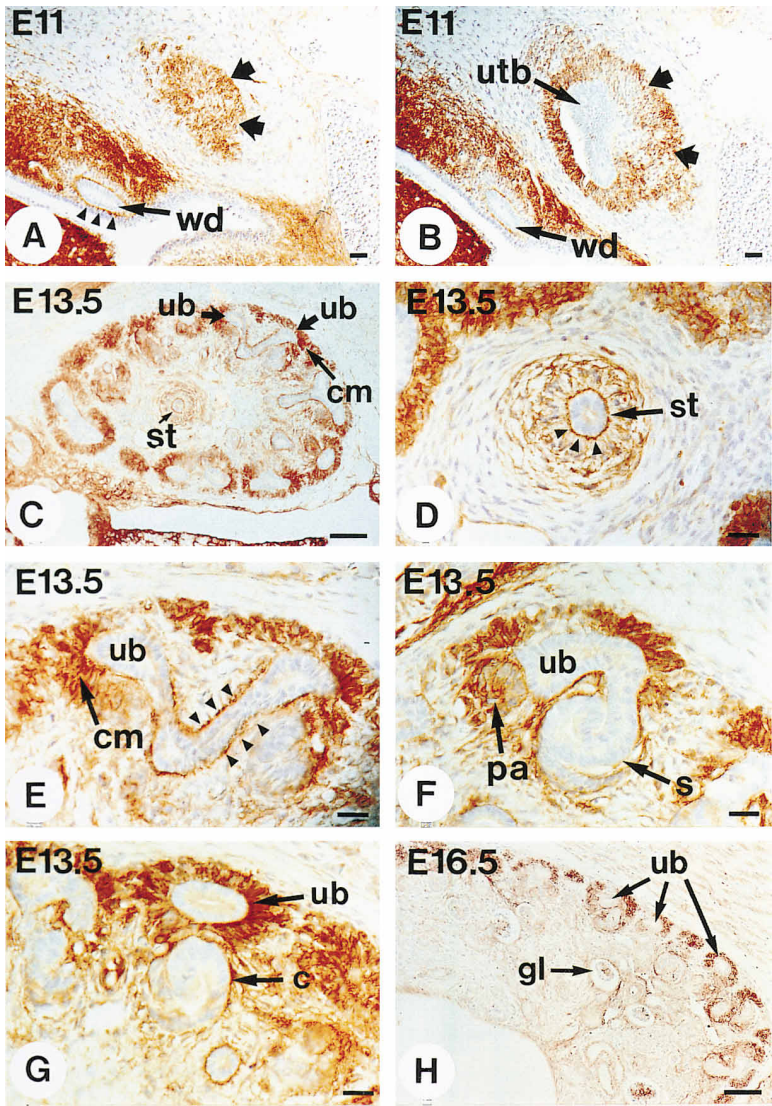


Figure 2. Immunohistochemical Localization of the Integrin $\alpha 8$ Subunit in the Developing Kidney

Sections of wild-type embryos were stained with antibody to the extracellular domain of the integrin $\alpha 8$ subunit. Abbreviations as in Figure 1.

(A and B) At E11, expression of the $\alpha 8$ subunit is evident in mesenchymal cells surrounding the Wolffian duct, with high concentrations at the interface between the duct epithelium and the surrounding mesenchymal cells (arrowheads). The $\alpha 8$ subunit is also expressed in mesenchymal cells above the tip of the growing ureteric bud (A, wide arrows) and surrounding the ureteric bud (B, wide arrows). (C–G) At E13.5, the $\alpha 8$ subunit is expressed in the condensing mesenchyme surrounding the tips of the branching ureter (C and E) and in pretubular aggregates (F) but not in comma-shaped bodies (G) or S-shaped (s) bodies (F). The $\alpha 8$ subunit is expressed at high levels in mesenchymal cells bordering on the ureteric epithelium, as shown in a transverse section (D, arrowheads) and longitudinal section (E, arrowheads) through the ureter.

(H) At E16.5, expression of the $\alpha 8$ subunit is seen in the outer layer of the kidney on condensing mesenchymal cells but not more internally on differentiated structures such as the glomerulus (gl).

Scale bars, 100 μm (C and H) and 10 μm (A, B, and D–G).

were evident (26% of the mutants; Figures 4C and 4D). In fact, the latter structures were similar in shape but typically smaller than wild-type kidneys (data not shown). In a few mutant animals (6%), two ureters had formed, had invaded the metanephric mesenchyme, and had branched to varying degrees. In a preliminary analysis, we did not observe major abnormalities in organs other than the kidneys.

Table 1. Kidney Deficiencies in Newborn Animals

Genotype	+/+	+/-	-/-
0 Kidneys	0	0	31
1 Rudiment	0	0	8
1 Kidney	0	0	15 ^a
Kidney + rudiment	0	0	2 ^a
2 Kidneys	30	105	1 ^a

The urogenital tract was dissected from newborn animals derived from intercrosses of mice heterozygous for a mutant $\alpha 8$ allele. The number of animals with the indicated number of kidneys or kidney rudiments was determined, and the genotype of the offspring was confirmed by Southern blot analysis.

^a Mutant kidneys were in most cases substantially smaller than wild-type kidneys.

Some of the homozygous mutant animals survived for several weeks or months after birth. All surviving animals had one kidney, suggesting that the cause of death in mutant animals was kidney agenesis or dysgenesis. The presence of animals with single kidneys demonstrates that stochastic factors contribute to the variability in phenotype. To test for potential genetic contributions, we interbred homozygous mutants that had survived for several months. Sixty-five of the 97 offspring (67%) of these crossings survived with one or two small kidneys. Because the original heterozygous founders are a genetic mix of 129 and C57Bl/6 mice, the data suggest that a genetic modifier locus (or multiple loci) present in one or both of the two genetic backgrounds modulates the penetrance of the $\alpha 8$ -dependent phenotype (see Discussion).

Integrin $\alpha 8 \beta 1$ Is Required for Growth and Branching of the Ureter and for Development of Epithelial Specializations

As discussed above, we showed that expression of the integrin $\alpha 8$ subunit is induced in mesenchymal cells bordering on the growing and branching ureteric bud and

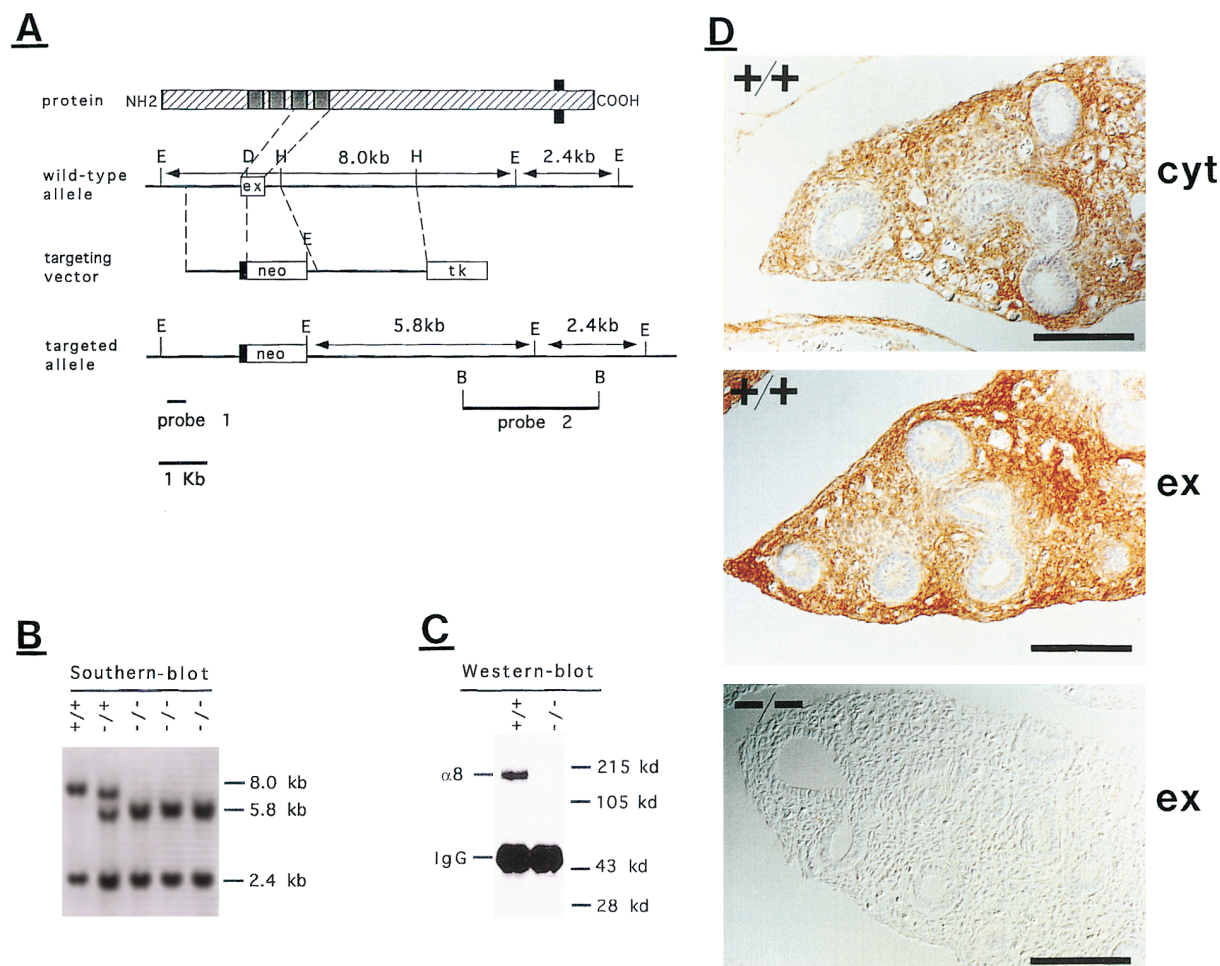


Figure 3. Targeting of the $\alpha 8$ Locus

(A) Schematic representation of the $\alpha 8$ protein and restriction map of the genomic $\alpha 8$ clone. The configuration of the targeting construct, the targeted allele, the probes used for screening, and the expected Southern blot fragments are indicated. E, EcoRI; H, HindIII; D, DpnI; B, BamHI. For DpnI only one of many restriction sites is shown.

(B) Mouse tail DNA from offspring of an intercross between heterozygous mice was digested with EcoRI and analyzed by Southern blotting with probe 2. The 8.0 kb band reflects the wild-type $\alpha 8$ allele, and the 5.8 kb band reflects the targeted $\alpha 8$ locus. A 2.4 kb band is detected in both genotypes.

(C) Extracts were prepared from lung tissue of newborn wild-type or homozygous mutant mice. $\alpha 8$ protein was immunoprecipitated with an antiserum specific for the extracellular domain of $\alpha 8$, followed by western blot with a second antiserum against the extracellular domain. Proteins were visualized by chemiluminescence. The positions of the $\alpha 8$ protein and the immunoglobulin G heavy chain are indicated.

(D) Sections through the lung of wild-type and homozygous mutant P0 animals were stained with an antibody against the cytoplasmic (cyt) or extracellular (ex) domain of integrin $\alpha 8$. Scale bars, 50 μ m.

on condensing mesenchymal cells in the metanephric mesenchyme. The expression patterns suggested that integrin $\alpha 8\beta 1$ may be required for formation, growth, and branching of the ureter; for formation of epithelial structures from mesenchymal cells; or for both. To distinguish among these possibilities, we analyzed by standard histological techniques the progressive development of the kidney in $\alpha 8$ -deficient embryos (Figure 5 and Table 2). We focused on E11.5, when the ureteric bud invades the metanephric mesenchyme, and on E13.5 and E16.5, when the ureter in wild-type animals has branched within the mesenchyme and signs of epithelial differentiation are visible.

In all wild-type and heterozygous animals, the ureteric bud had invaded the metanephric mesenchyme at E11.5

(Figure 5A). In contrast, in all $\alpha 8$ -deficient mice, the ureteric bud had formed but had not yet invaded the metanephric mesenchyme (Figure 5B). No defects were observed in the development of the mesonephros or the Wolffian duct (data not shown). At E13.5 in wild-type animals, the ureter had branched and epithelialization of mesenchymal cells was progressing (Figure 5C); in homozygous mutant animals, kidney development was affected to varying degrees (Figures 5D–5F and Table 2). In 54% of the developing nephric systems, the metanephric mesenchyme was degenerating and no or only a short ureter was visible (Figure 5F). In the remaining cases, a ureter had invaded the metanephric mesenchyme, but branching and formation of epithelial specializations were evident in only 18% of cases (Figures

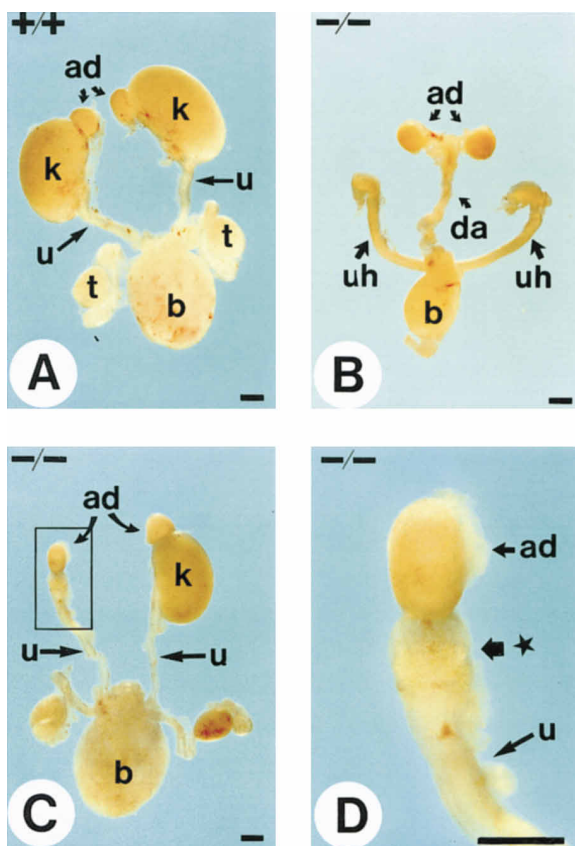


Figure 4. Kidney Phenotype in $\alpha 8$ -Deficient Mice at P0
The urogenital tract was dissected from wild-type (A) or $\alpha 8$ -deficient (B–D) mice.
(A) In wild-type mice the adrenal glands (ad), kidneys (k), ureter (u), bladder (b), and testes (t) were well developed.
(B) The most seriously affected mutants had no ureter and kidney, but the bladder and adrenal glands were unaffected. Note that the dorsal aorta (da) and uterine horns (uh) were not removed in this dissection.
(C) One small kidney and one rudiment (boxed) from a mutant animal.
(D) High magnification view of the rudiment (boxed in C). The star indicates the undifferentiated mesenchyme between the adrenal gland and the ureter. Scale bars, 100 μ m.

5D–5F). Branching and epithelialization was less substantial than in wild-type or heterozygous littermates (compare Figure 5C to Figures 5D and 5E).

We conclude that in the absence of integrin $\alpha 8 \beta 1$ the ureteric bud forms properly, but growth of the ureteric bud toward the metanephric mesenchyme and growth and branching of the ureteric epithelium within the metanephric mesenchyme are defective. In addition, recruitment of mesenchymal cell into epithelial structures is impaired.

Expression of p75 and Pax-2 Is Induced in Metanephric Mesenchyme of Mutant Animals

Conversion of the metanephric mesenchyme to epithelial structures is impaired in mutant animals. One explanation may be that the delayed growth of the ureter leads to delayed invasion of the mesenchyme at a time when it has lost its competence to respond to inductive

signals or the signals are no longer present. Alternatively, integrin $\alpha 8 \beta 1$ may have a direct role in regulating migration of mesenchymal cells away from the ureteric epithelium after they have received the inductive stimulus. Finally, $\alpha 8 \beta 1$ may have a role in the epithelialization process itself. To distinguish among these possibilities, we analyzed the expression of markers for early inductive events within the mutant mesenchyme (Figure 6). We focused on the transcription factor Pax-2 and the low affinity neurotrophin receptor p75^{NTR}, both of which are induced in the condensing mesenchyme. Pax-2 is also expressed in the ureteric epithelium and p75^{NTR} in differentiating renal vesicles (Figure 6; Dressler et al., 1990; Sariola et al., 1991).

Induction was evaluated in 14 mutant mesenchyme between E13.5 and E15.5. The ureteric bud had invaded the mesenchyme in 5 cases. Expression of the markers was induced in all 5 mesenchyme that had been invaded by the ureter, and it was evident in cells that were not in direct contact with the ureter (Figures 6B and 6D). We conclude that the mutant metanephric mesenchyme was able to respond to ureter-derived inductive signals and that mesenchymal cells were migrating after induction. Formation of mesenchymal condensates was also evident, but the kidney rudiments appeared less well organized than wild-type kidneys, and few if any comma-shaped and S-shaped bodies were visible (compare Figures 6A and 6C to Figures 6B and 6D). While at E13.5 the ureter had invaded 47% of the mutant mesenchyme and induction of Pax-2 and p75^{NTR} was evident in all mesenchyme invaded by the ureter, only 17% of the animals showed at birth signs of epithelialization of metanephric mesenchymal cells (Table 2 and Figure 6). Thus we conclude that in $\alpha 8$ -deficient animals, epithelialization of mesenchymal cells is defective.

The Developing Kidney Contains a Novel Ligand for Integrin $\alpha 8 \beta 1$

Integrin $\alpha 8 \beta 1$ serves as a receptor for FN, VN, and TN-C, but none of these ligands is expressed in the correct patterns to account for the kidney phenotype in $\alpha 8$ -deficient mice (see Discussion). To characterize the distribution of potential $\alpha 8 \beta 1$ ligands, we stained whole-mount kidneys and kidney sections with a truncated recombinant integrin $\alpha 8 \beta 1$ heterodimer tagged with alkaline phosphatase ($\alpha 8 \beta 1$ -AP). Similar chimeras have been generated with receptor tyrosine kinases to identify their ligands (Cheng and Flanagan, 1994). To obtain this heterodimer, the extracellular domain of the integrin $\beta 1$ subunit was fused to an alkaline phosphatase tag that replaced the transmembrane and cytoplasmic domains. To facilitate purification, the extracellular domain of the $\alpha 8$ subunit was expressed with a C-terminal polyhistidine-myc tag. Expression vectors containing these chimera were cotransfected into COS cells and secreted $\alpha 8 \beta 1$ -AP heterodimers were purified from the supernatants. In enzyme-linked immunosorbent assays, the $\alpha 8 \beta 1$ -AP heterodimers bound specifically to FN, VN, and TN-C, the three known ligands for integrin $\alpha 8 \beta 1$ (S. D. et al., unpublished data).

Whole-mount kidneys were stained with antibodies to the integrin $\alpha 8$ subunit or with $\alpha 8 \beta 1$ -AP. As predicted,

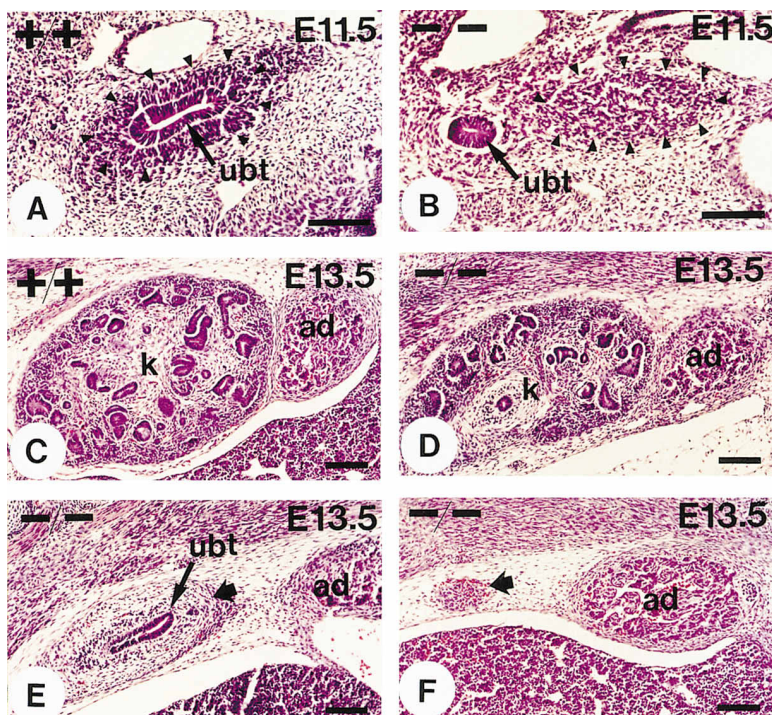


Figure 5. Kidney Phenotype in $\alpha 8$ -Deficient Mice at E11.5 and E13.5

Sections were stained with hematoxylin and eosin.

(A and B) Sections from E11.5 wild-type and mutant kidney regions. The arrowheads demarcate the metanephric mesenchyme. In wild-type animals (A) the ureteric bud (ubt) had invaded the metanephric mesenchyme. In $\alpha 8$ -deficient mice (B) the ureteric bud had developed but had not invaded the metanephric mesenchyme.

(C) Section from a wild-type metanephros at E13.5.

(D-F) Sections showing the extent of metanephric development at E13.5 in $\alpha 8$ -deficient animals. In some animals the ureter had branched within the mesenchyme (D); in some it had invaded the mesenchyme (wide arrow) but had not branched (E); and in others it had not reached the mesenchyme (wide arrow), which was in the process of degeneration (F).

k, kidney; ad, adrenal gland. Scale bars, 100 μ m.

the $\alpha 8$ antibody stained condensing mesenchymal cells surrounding ureteric branches on the outside of the kidney (Figure 7A). The soluble recombinant $\alpha 8\beta 1$ -AP heterodimer revealed a complementary pattern (Figure 7B), staining the tips of the branching ureter. In prolonged staining reactions, additional diffuse staining was observed over most kidney mesenchymal cells (data not shown). The diffuse staining was most likely due to interaction of $\alpha 8\beta 1$ -AP with its ligands FN and TN-C. Less pronounced staining for these ligands may reflect their diffuse distribution, may indicate low expression levels, or may be a consequence of the stringent fixation and washing conditions during the staining procedure, which may disrupt some but not all of the receptor-ligand interactions. Colocalization of integrin $\alpha 8\beta 1$ with a novel ligand was also evident in kidney sections where $\alpha 8\beta 1$ -AP stained the surface of the branching ureteric epithelium (Figures 7D, 7F, and 7H), in an overlapping but more confined pattern compared with that seen using antibodies against the integrin $\alpha 8$ subunit (Figures 7E and 7G). Staining with $\alpha 8\beta 1$ -AP was specific, as it was blocked by an antibody to the $\beta 1$ chain but not by

a control antibody (data not shown). In agreement with our earlier observations that integrin $\alpha 8\beta 1$ binds in a cation-dependent manner to an RGD motif within its ligands (Müller et al., 1995), staining was also dependent on divalent cations and completely blocked by RGD but not RGE peptides (Figures 7I and 7K).

To provide further evidence for a novel $\alpha 8\beta 1$ ligand, we analyzed extracts from embryonic and adult kidneys by Far-Western blot using $\alpha 8\beta 1$ -AP as a probe (Figure 7L). Proteins with molecular weights of about 220, 150, 110, and 60–90 kDa were detected in extracts from embryonic kidney. Very low amounts of the 60–90 kDa species were also present in adult kidney. Interactions of $\alpha 8\beta 1$ -AP with these proteins were abolished by RGD but not RGE peptides (data not shown). Western blot analysis suggested that the 220 kDa species was most likely FN (Figure 7L). However, the 150 and 110 kDa proteins and at least some of the 60–90 kDa proteins did not comigrate with FN, VN, or TN-C (Figure 7L and data not shown). We conclude that the embryonic kidney contains novel ligands for integrin $\alpha 8\beta 1$. Because some of these proteins are expressed in embryonic but not adult kidney, they are strong candidates for mediators of integrin $\alpha 8\beta 1$ functions during kidney development.

Discussion

Integrin $\alpha 8\beta 1$ Is Critically Important for Epithelial-Mesenchymal Interactions during Kidney Morphogenesis

We demonstrate here that integrin $\alpha 8\beta 1$ plays a crucial role in epithelial-mesenchymal interactions during kidney morphogenesis. Expression of integrin $\alpha 8\beta 1$ is induced in mesenchymal cells bordering on the growing and branching ureter epithelium. Inactivation of the $\alpha 8\beta 1$ receptor in mice leads to deficits in growth of the ureteric bud as it extends toward the metanephric mesenchyme

Table 2. Analysis of Metanephric Development

Developmental age	E11.5	E13.5	P0
Wild type	n = 10	n = 10	n = 60
Ureter, branches	100%	100%	100%
$\alpha 8$ Mutant	n = 8	n = 28	n = 114
No ureter	0%	29%	75%
Ureter, not in mesenchyme	100%	25%	0%
Ureter, in mesenchyme	0%	29%	9%
Ureter, branches	0%	18%	17%

Wild-type or mutant embryos (E11.5 or E13.5) were sectioned, stained with hematoxylin and eosin, and analyzed for progression of metanephric development. P0 animals were analyzed by dissection and visual inspection.

n, number of ureters analyzed.

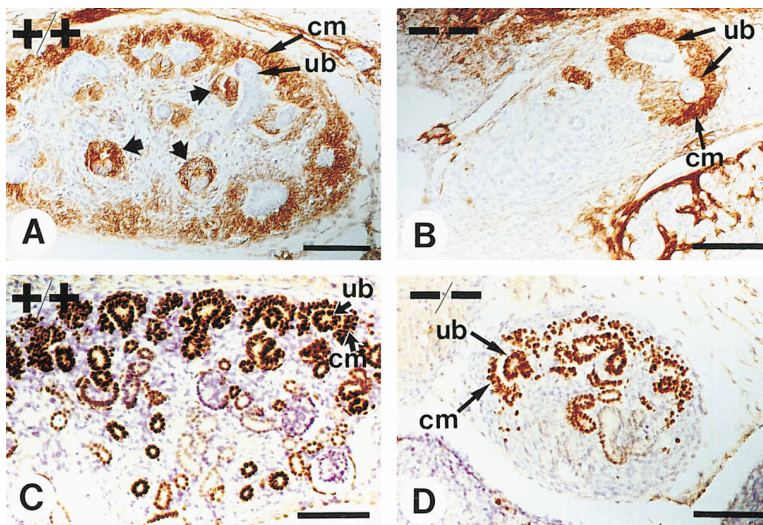


Figure 6. Analysis of p75^{NTR} and Pax-2 Expression at E13.5 and E15.5

(A and B) Sections from E13.5 wild-type (A) and mutant (B) animals were stained with antibodies to the p75^{NTR} receptor. Expression was apparent in wild-type and mutant animals in condensing mesenchymal cells (cm) surrounding the ureter branches (ub). In wild-type animals, p75^{NTR} expression was also observed in renal vesicles (A, wide arrows). (C and D) Sections from E15.5 wild-type (C) and mutant (D) animals were stained with antibodies to Pax-2, which is expressed both in the ureteric epithelium and upon induction in the condensing mesenchyme. Only a small part of a section from the wild-type kidney is shown, whereas the whole rudiment of an $\alpha 8$ -deficient animal is visible. Note that in mutant animals the ureter had formed very few branches but Pax-2 expression was induced. Scale bars, 50 μ m.

and to reduced branching of the ureteric epithelium within the metanephric mesenchyme. In addition, epithelialization of the metanephric mesenchyme is impaired. By using an $\alpha 8 \beta 1$ -AP chimera, we provide evidence for a novel ligand coexpressed with $\alpha 8 \beta 1$ at the border between epithelial and mesenchymal cells. This ligand is a strong candidate for mediator of the effects of mesenchymally expressed integrin $\alpha 8 \beta 1$ on ureter development and kidney morphogenesis.

Animals lacking integrin $\alpha 8 \beta 1$ are born with variable kidney phenotypes: some lack kidneys and ureters; some have ureters attached to kidney rudiments; and others have one or two ureters attached to small, functional kidneys. The latter can survive to adulthood. Analysis of the establishment of the phenotype during development provides an explanation for this variability. At E11.5, when the ureteric bud has formed and has initiated invasion of the metanephric mesenchyme in wild-type embryos, both the ureteric bud and metanephric mesenchyme are present, but invasion has not commenced in mutants. In some mutants, invasion is never successfully initiated, leading to renal agenesis and ureteric bud regression; in others, invasion is initiated at later times, but branching morphogenesis of the ureter and epithelialization of the metanephric mesenchyme are severely compromised. Thus, in many mutants, only kidney rudiments, consisting of undifferentiated mesenchyme attached to the ureter, are present at birth; in others, morphogenesis at a reduced rate generates small kidneys.

It is likely that the variability in the manifestation of the phenotype is partially a consequence of differences in the genetic background of the animals analyzed. Individual $\alpha 8$ -deficient mice contain varying chromosomal contributions from both 129 and C57Bl/6 inbred mice. Some parameter of ureter growth such as replication rate of epithelial cells may vary in different genetic backgrounds. In support of a genetic contribution, 67% of the offspring of intercrosses of homozygous mutant survivors developed one or two kidneys (in contrast to 18% in the crosses between heterozygous animals). In the future, it will be necessary to cross the $\alpha 8$ allele into different genetic backgrounds to map potential modifier loci.

Adhesive Interactions Mediated by Integrin $\alpha 8 \beta 1$

It is well established that branching morphogenesis of epithelial cell sheets in developing organs is highly dependent on interactions with surrounding mesenchymal cells (reviewed in Grobstein, 1956; Saxen, 1987; Gurdon, 1992; Ekblom, 1996). When the ureteric epithelium is cultivated *in vitro* in the absence of mesenchyme, it flattens and spreads and no tubular growth occurs (reviewed by Grobstein, 1956). Tubular growth and branching are observed in cocultures with mesenchymal cells, and both proliferation and branching of the ureteric bud are affected by local environmental factors (Grobstein, 1953; reviewed in Saxen, 1987). An influence of the surrounding mesenchyme on epithelial growth has also been demonstrated for the Wolffian duct. Holtfreter (1944) suggested that the mesenchyme may guide the forming duct while it extends rostrocaudally. Subsequent transplantation experiments have shown that duct formation and growth depend on adhesive interactions between duct cells and mesenchymal cells (Ti-Chow-Tung and Su-Hwei-Ku, 1944; Poole and Steinberg, 1981, 1982).

Results presented above demonstrate that integrin $\alpha 8 \beta 1$ is one of the molecules that mediates interactions between the ureter and mesenchyme. It has previously been shown that integrin $\alpha 8 \beta 1$ binds to FN, VN, and TN-C (Müller et al., 1995; Schnapp et al., 1995; Varnum-Finney et al., 1995). None of these molecules exhibits an appropriate pattern of spatiotemporal expression to account for the kidney phenotype of $\alpha 8$ -deficient mice. FN is expressed in the metanephric mesenchyme prior to invasion by the ureteric bud and is down-regulated upon induction (Ekblom, 1981; Aufderheide et al., 1987). VN is not expressed in the embryonic kidney (Seiffert et al., 1991). TN-C expression is first evident at E13.5 and is restricted to early stages of the stromal cell differentiation pathway. Its expression increases thereafter, much too late to account for the phenotype in $\alpha 8 \beta 1$ -deficient animals (Aufderheide et al., 1987). Consistent with these observations, mice with targeted mutations in the VN or TN-C genes develop without kidney abnormalities (Saga et al., 1992; Zheng et al., 1995). Embryos homozygous for a targeted mutation in the FN gene die too early to analyze kidney development, but antibodies

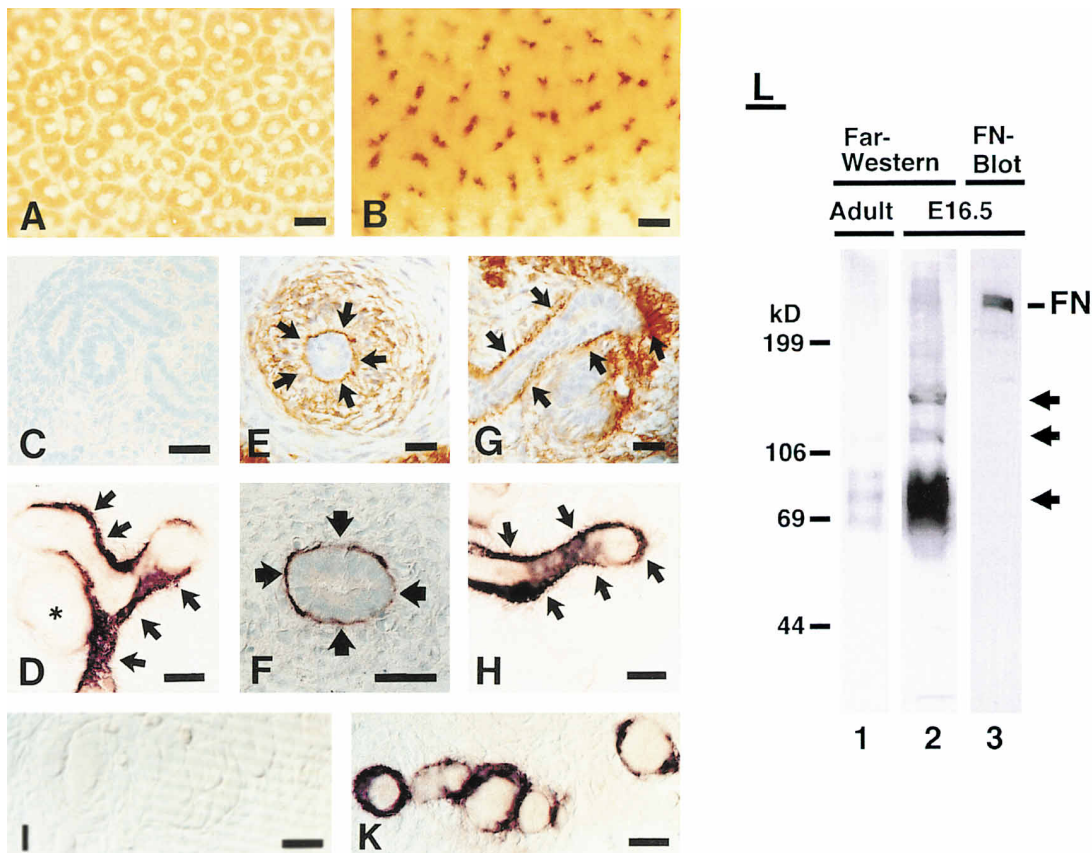


Figure 7. Expression of a Novel Integrin $\alpha 8\beta 1$ Ligand within the Kidney

(A and B) Whole-mount kidneys were stained with an antibody specific for the extracellular domain of integrin $\alpha 8$ (A) or with $\alpha 8\beta 1$ -AP (B). (C–I and K) Sections from an E13.5 kidney were stained with a control AP fusion protein (C), with an antibody to $\alpha 8$ (E and G), or with $\alpha 8\beta 1$ -AP (D, F, H, I, and K). Staining was carried out in the presence of RGD (I) or RGE (K) peptides. The arrows indicate areas where colocalization between $\alpha 8\beta 1$ and its novel ligand is evident. The asterisk in (D) marks a comma-shaped body. (L) Extracts were prepared from embryonic and adult kidneys. Proteins were separated on 6% polyacrylamide gels, transferred to nitrocellulose, and probed with either $\alpha 8\beta 1$ -AP or an antibody to FN. The arrows indicate novel proteins that interact in an RGD-dependent manner with $\alpha 8\beta 1$ -AP. Scale bars, 50 μ m (A and B) and 10 μ m (C–K).

to FN do not perturb kidney development in organ cultures (Klein et al., 1988; Sariola et al., 1988; George et al., 1993). Abundant binding sites for a soluble $\alpha 8\beta 1$ -AP chimera were localized to the interface between the branching ureter and surrounding mesenchymal cells, suggesting that a novel ligand for integrin $\alpha 8\beta 1$ is appropriately localized to mediate the interactions essential for normal kidney morphogenesis. Candidate ligand(s) expressed in embryonic but not adult kidney have also been identified in blots using $\alpha 8\beta 1$ -AP as a probe. These ligand(s) may mediate adhesive interactions between the ureter epithelium and mesenchymal cells expressing integrin $\alpha 8\beta 1$. In the absence of integrin $\alpha 8\beta 1$, adhesive interactions that are required for growth and branching of the ureter epithelium may be disrupted, leading to a reduced rate of kidney morphogenesis and renal agenesis or dysgenesis.

Participation of Integrin $\alpha 8\beta 1$ in Inductive Interactions

In the simplest model of the reciprocal interactions that mediate kidney morphogenesis, formation of the metanephric kidney involves two signals, one derived from

the metanephric mesenchyme inducing growth and branching of the ureter, and a second derived from the ureter inducing epithelialization of the mesenchyme. Integrins activate cellular signal transduction mechanism upon ligand binding and regulate actin cytoskeletal organization and dynamics (reviewed in Clark and Brugge, 1995; Schwartz et al., 1995). It is therefore likely that integrin $\alpha 8\beta 1$ plays a direct role in the signaling systems that coordinate development of epithelial and mesenchymal cells. In the following sections we discuss the role of integrin $\alpha 8\beta 1$ in epithelial–mesenchymal interactions in light of the signaling systems that are likely to be disrupted in $\alpha 8\beta 1$ -deficient animals.

Disruption of Metanephric Mesenchyme–Derived Inductive Signals

The earliest abnormality observed in $\alpha 8\beta 1$ -deficient mice is decreased growth of the ureteric bud, suggesting a deficit in the transduction of a signal derived from the metanephric mesenchyme. Recent experiments in organ culture and analyses of mice carrying targeted mutations have shown that GDNF is one of the mesenchyme-derived secreted signals required for

growth and branching of the ureter. Activation of the c-ret receptor tyrosine kinase in ureteric epithelial cells mediates the response of these cells to GDNF (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1996; Vega et al., 1996). This is apparently not the only signaling system inducing ureteric bud growth, because a subset of mice lacking c-ret still form a ureter (Schuchardt et al., 1996). Strikingly, in both $\alpha 8$ - and c-ret-deficient mice, similar phenotypic variability is apparent, ranging from lack of kidneys to small kidneys. At this point it is not known whether defects in ureteric bud growth in $\alpha 8 \beta 1$ -deficient mice reflect disruption in the GDNF/c-ret signaling cascade, inhibition of ureteric bud responsiveness to this cascade, or disruption of another signaling cascade that accounts for residual kidney morphogenesis in c-ret mutants.

It was not anticipated that integrin $\alpha 8 \beta 1$ receptor expressed by mesenchymal cells is involved in signaling from the mesenchyme to the ureteric bud. Recent results obtained in kidney organ cultures may provide an explanation. Antisense oligonucleotides against c-ret not only reduce levels of c-ret expression in the ureter and disrupt branching morphogenesis; they also lead to a drastic reduction in the expression of numerous ECM molecules such as laminin, collagen IV, and perlecan (Liu et al., 1996). As one possible scenario, in c-ret-deficient animals, expression of an $\alpha 8 \beta 1$ ligand may be absent; in $\alpha 8$ mutants, the ligand may not be appropriately assembled into a basement membrane. Since the ECM has been shown to enhance efficiency of growth factor signaling pathways by immobilization or presentation of these factors or by activation of synergistic cosignaling pathways (reviewed in Clark and Brugge, 1995; Schwartz et al. 1995), ECM disorganization in the $\alpha 8 \beta 1$ mutant animals may disrupt transmission of inductive signals, disrupting proliferative or migratory responses of ureteric bud epithelial cells.

Disruption of Ureter-Derived Inductive Signals

The impaired epithelialization of the metanephric mesenchyme in $\alpha 8$ mutants suggests that $\alpha 8 \beta 1$ may also regulate signal transmission from the ureteric bud to the metanephric mesenchyme. Although little is known about the signaling molecules involved, it is clear that induction requires close proximity between the ureteric epithelium and metanephric mesenchyme (reviewed in Saxen, 1987). Subsequently, cells that have received the inductive signal migrate away from the ureteric epithelium, leading to spreading of the signal over several cell diameters (Saxen, 1987). Potential candidates for the inductive signal include members of the wnt and TGF- β superfamilies (reviewed in Parr and McMahon, 1994; Roelink, 1996). Both wnt-11 and BMP7 are expressed in the developing kidney, and wnt-11 is concentrated in the tip of the ureteric bud (Lyons et al., 1995; Kispert et al., 1996). Mice with targeted mutations in BMP7 and wnt-4 show defects in kidney development (Stark et al., 1994; Dudley et al., 1995; Luo et al., 1995). Integrin $\alpha 8 \beta 1$ is unlikely to mediate initial inductive events because expression of this integrin within the mesenchyme is an early response to ureter-derived inducers. Indeed, we have strong evidence that some

initial signals are transmitted, because both Pax-2 and p75^{NTR} are induced in mutant mesenchymal cells. Integrin $\alpha 8 \beta 1$ also is not essential for subsequent migration of mesenchymal cells, because expression of Pax-2 and p75^{NTR} is observed in mesenchymal cells several cell diameters away from the ureter.

Still, morphogenetic events following induction are defective in integrin $\alpha 8 \beta 1$ -deficient mice. This is most strikingly demonstrated by the observation that in some mutant animals an unbranched ureter attached to an undifferentiated mesenchyme is visible at birth or even months after birth. Integrins play a central role in ECM assembly, and these matrices are essential to generate the polarity of epithelial and endothelial cells (reviewed in Ekblom, 1996). Organ culture experiments have shown that formation of polarized epithelia from the kidney mesenchyme is dependent on proper basement membrane assembly, a process that is dependent on integrin $\alpha 6 \beta 1$ expressed in the newly generated epithelial cells (reviewed in Ekblom, 1996). It is conceivable that integrin $\alpha 8 \beta 1$ plays a role in early steps of matrix assembly and that it may exert its effect on epitheliogenesis by helping to organize a matrix that localizes short-range signaling molecules such as wnts and BMPs.

The activation of $\alpha 8$ expression in the mesenchyme by the ureter is presumably caused at the transcriptional level. Transcription factor Pax-2 is also induced in the metanephric mesenchyme upon contact with the ureter (Dressler et al., 1990), and Pax-2 is required for kidney development (Torres et al., 1995). This raises the possibility that Pax-2 may regulate $\alpha 8$ expression. Future analysis of the mechanism that leads to induction of $\alpha 8$ within mesenchymal cells should help in the identification of the inductive signal and its mode of action.

A General Role for Integrins in Epithelial-Mesenchymal Interactions

The data presented here strongly support the possibility that integrin $\alpha 8 \beta 1$ plays a critical role in epithelial-mesenchymal interactions. It is well established that growth of epithelial tubules and branching morphogenesis of epithelial cell sheets in forming organs are dependent on epithelial-mesenchymal interactions. During embryogenesis, integrin $\alpha 8 \beta 1$ is expressed in the mesenchyme of many duct-forming organs. However, no major deficits were observed in $\alpha 8 \beta 1$ -deficient mice in other tissues besides the kidney. It is conceivable that $\alpha 8 \beta 1$ functions in other tissues are masked by additional members of the integrin family that may also regulate epithelial-mesenchymal interactions. Indeed, $\alpha 6$ -containing integrins regulate branching morphogenesis in the submandibular gland in organ culture (Kadoya et al., 1995). Mice carrying a targeted mutation in the integrin $\alpha 3$ gene have altered branching of glomerular capillary loops and of bronchi (Kreidberg et al., 1996). In *Caenorhabditis elegans*, mutations within an integrin β -chain gene lead to apparent deficits in epithelialization of distal tip cells and ovarian sheet cells (Gettner, 1994). Future analyses of mice carrying targeted mutations in multiple integrin α genes will be important for directly investigating potential redundant integrin functions in epithelial-mesenchymal interactions.

Experimental Procedures

DNA Constructs

A cDNA encoding the mouse $\alpha 8$ protein was isolated (S. D. et al., unpublished data) and sequenced. An $\alpha 8$ genomic clone was isolated by screening a 129SVJ genomic library (Stratagene) with a mouse $\alpha 8$ cDNA probe. DNA fragments containing $\alpha 8$ exons were identified by DNA sequencing. An exon encoding amino acids 362–424 was interrupted by the *PGK-neo* selectable marker (Figure 2A). A genomic $\alpha 8$ DNA fragment extending from the *SacI* site to the *DpnI* site was isolated and shortened from the *SacI* site by 200 bp with *Bal31*, Klenow fragment blunted, and fused to the Klenow-blunted *HindIII* site in *PGK-neo* to generate *PGK-neo-1.2*. A 3.1 kb *HindIII* genomic DNA fragment 3' of the $\alpha 8$ exon was inserted into the *HindIII* site of *PGK-tk*, and an *XhoI* fragment containing the *HindIII* fragment and the *PGK-tk* expression cassette was cloned into the *XhoI* site of *PGK-neo-1.2*. For Southern blots, probe 1 was obtained by subcloning a 200 bp genomic 5' to the the targeting vector, and probe 2 was obtained by subcloning a genomic *BamHI* fragment near the 3' end (Figure 2A).

Cloning of $\beta 1$ -AP and $\alpha 8$ -HIS-myc, COS-cell expression, and protein purification will be described elsewhere (S. D. et al., unpublished data).

Generation of $\alpha 8$ -Deficient Mice

The JM1 embryonic stem cells (J. J. M. and R. A. P., unpublished data) were grown on mitotically inactivated STO cells and electroporated with 25 μ g of linearized targeting vector. After 8–10 days in selective medium (300 μ g/ml G418 and 0.2 μ M FIAU), colonies were picked, expanded, and screened by polymerase chain reaction and Southern blot analysis and used for injection of C57Bl/6 blastocysts (Joyner, 1993). To obtain germline transmission, chimeric male mice were mated to C57Bl/6 females.

Histological Methods

Histological methods were carried out as described previously (Jones et al., 1994). Primary antibodies were used as follows: affinity-purified antibody to the $\alpha 8$ extracellular or cytoplasmic domains at 10 and 25 μ g/ml, respectively; to p75^{NTR} at 10 μ g/ml (Weskamp and Reichardt, 1991); and to Pax-2 at 15 μ g/ml (a gift from G. Dressler).

For whole-mount staining with $\alpha 8\beta 1$ -AP, kidneys were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS); blocked with 1% bovine serum albumin (BSA), 0.1% Triton X-100 in PBS; washed with buffer J (0.1% BSA, 100 mM NaCl, 1 mM $MnCl_2$, 0.1% Triton X-100, 25 mM Tris-HCl [pH 7.5]); and incubated with 3 μ g/ml $\alpha 8\beta 1$ -AP in buffer J. Where indicated, GRGDSP or GRGESP peptides (50–100 μ g/ml) were included. Samples were washed three times with buffer J, once with 100 mM NaCl, 1 mM $MnCl_2$, 0.1% Triton X-100, 25 mM Tris-HCl (pH 7.5); fixed with 60% acetone, 3% formaldehyde in 20 mM HEPES (pH 7.0); washed with 150 mM NaCl, 20 mM HEPES (pH 7.0); heated for 1 hr at 65°C; rinsed with AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl [pH 9.5]); and developed with substrate solution (0.17 mg/ml 5-bromo-4-chloro-3-indolylphosphate, 0.33 mg/ml nitro blue tetrazolium in AP buffer). For antibody staining, fixed samples were blocked with 1% BSA, 0.1% Triton X-100, and 1% H_2O_2 in PBS; washed with buffer D (1% BSA, 0.1% Triton X-100, 0.02% sodium azide in PBS); incubated with 10 μ g/ml $\alpha 8$ antibody; washed with buffer D; and detected by the ABC method (Vector). Staining of sections with $\alpha 8\beta 1$ -AP will be described elsewhere (S. D. et al., unpublished data).

Far-Western Blots

Kidneys were homogenized in PBS in a dounce homogenizer and lysed in 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl (pH 8.0). Proteins were separated on 6% SDS-polyacrylamide gels. After transfer of protein to nitrocellulose, filters were blocked for 1 hr with 3% BSA in PBS and developed as described above for tissue sections.

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